Distinct Mechanisms of Epithelial Adhesion for *Candida albicans* and *Candida tropicalis*

Identification of th Participating Ligands and Development of Inhibitory Peptides

Catherine M. Bendel* and Margaret K. Hostetter,*‡
With the technical assistance of Mark McClellan
Departments of *Pediatrics and ‡Microbiology, University of Minnesota, Minneapolis, Minnesota 55455*

Abstract

The yeast *Candida albicans* is the leading cause of disseminated fungal infection in neonates, immunocompromised hosts, diabetics, and postoperative patients; *Candida tropicalis* is the second most frequent isolate. Because the integrin analogue in *C. albicans* shares antigenic, structural, and functional homologies with the β_2-integrin subunits α_M and α_X, we investigated the role of integrin analogues in epithelial adhesion of *C. albicans* and *C. tropicalis*.

On flow cytometry with the monoclonal antibody (mAb) OKM1, surface fluorescence was highest for *C. albicans* and significantly reduced for *C. tropicalis* ($P < 0.001$). However, adhesion to the human epithelial cell line HeLa S3 did not differ for these two candidal species: specific adhesion was highest for *C. albicans* at 44.0±1.8%, and only slightly lower for *C. tropicalis* at 38.8±3.6% (P = NS). The disparity between expression of the integrin analogue and epithelial adhesion suggested distinct mechanisms for this process in *C. albicans* versus *C. tropicalis*.

Preincubation of *C. albicans* with anti-α_M mAbs, with purified iC3b (the RGD ligand for the integrin analogue), or with 9–15-mer RGD peptides from iC3b all inhibited epithelial adhesion significantly ($P < 0.001$–0.04). Purified fibronectin or fibronectin-RGD peptides failed to block *C. albicans* adhesion. In contrast, epithelial adhesion of *C. tropicalis* was significantly inhibited by purified fibronectin and its RGD peptides ($P \leq 0.021$), but not by iC3b nor the iC3b-RGD peptides. Both iC3b and fibronectin were identified on the surface of epithelial cells after growth in serum-free medium. A polyclonal antibody to C3 inhibited *C. albicans* adhesion while a control antibody to fibronectin was ineffective; the converse was true for *C. tropicalis*. These results indicate that the pathogenic yeasts *C. albicans* and *C. tropicalis* recognize distinct RGD ligands present at the surface of the epithelial cell and that these interactions can be differentially inhibited by defined RGD peptides containing appropriate flanking sequences. (*J. Clin. Invest.* 1993. 92:1840–1849.) Key words: *Candida albicans* • *Candida tropicalis* • epithelium • integrins • RGD peptides.

Introduction

The yeast *Candida albicans* is the leading cause of disseminated fungal infection in the immunocompromised host, the diabetic, the neonate, and the postoperative patient. In representative studies of fungemia in each of these patient populations, *C. albicans* accounts for 60–80% of isolates and *C. tropicalis* for 12–20% of isolates, whereas other less pathogenic *Candida* species including *C. parapsilosis*, *C. glabrata*, and *C. krusei* are cumulatively identified < 20% of the time (1–6). Genetically related yeasts such as *Saccharomyces cerevisiae* are even less frequently implicated in invasive disease (7). Similar results obtain when colonizing flora are enumerated; in one series in cancer patients, *C. albicans* was present in 68% of isolates from sites other than blood, while *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, and *S. cerevisiae* accounted for 12.3%, 10.3%, 3.0%, 1.5%, and 0.8% of isolates, respectively (8). Thus, in a hierarchy of pathogenicity, the invasive potential of *C. albicans* and *C. tropicalis*—and the lesser virulence of related species—appears to reflect their relative abilities to colonize the human host.

The site of initial contact between *Candida* and the host is thought to be the epithelium of the gastrointestinal or genitourinary tract, and at least two studies have shown preferential adhesion by *C. albicans*, as opposed to other *Candida* species, to exfoliated buccal or vaginal epithelial cells (9, 10); a number of surface carbohydrates (e.g., those of ABO or Lewis^a blood group antigens) have been proposed as target molecules on the epithelial cell (11).

However, we thought it possible that protein–protein interactions might provide a more general mechanism of candidal adhesion. For example, the integrin analogue in *C. albicans* shares antigenic, structural, and functional homologies with α_M and α_X of the β_2-integrin subset. As previously demonstrated, iC3b-coated sheep erythrocytes and mAbs recognizing α_M bound specifically to both yeast- and hyphal-phase *C. albicans* (12–15). Immunodetection with the mAb anti-Mo1 identified a major band of M_r 165±15 kD after SDS-PAGE and Western blotting of *C. albicans* cytosolic extracts under nonreducing conditions, a mass consistent with that reported for α_M and α_X (16, 17). Moreover, C3 fragment iC3b, an identified ligand for $\alpha_M\beta_2$ and $\alpha_X\beta_2$ dimers, bound saturably and reversibly to *C. albicans* with a K_a virtually identical to that established for the binding of iC3b to these integrins on human neutrophils (14, 18).

In mammalian cells, the β_2-integrins also regulate the adhesion of leukocytes to endothelium by recognition of the tripep-

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Address reprint requests to Dr. Catherine M. Bendel, UMHC Box 296, University of Minnesota, 420 Delaware Street S.E., Minneapolis, MN 55455.

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tide sequence arginine-glycine-aspartic acid (RGD)[1] (19–21); RGD peptides have been shown to inhibit this interaction (22). We have previously demonstrated a similar function for the integrin analogue in *C. albicans* in mediating adhesion to human umbilical vein endothelium (23). In these earlier studies, the degree of surface expression of the integrin analogue in *C. albicans* correlated directly with adhesion to the endothelial monolayer; specific adhesion was inhibited by 50–80% with the use of mAbs recognizing α_M or with purified iC3b (23).

Given these intriguing homologies, we therefore sought to correlate surface expression of the integrin analogue with epithelial adhesion for the two most pathogenic yeasts, *C. albicans* and *C. tropicalis*, and to identify those components on the microorganism and on the epithelial cell which mediate this interaction. Assuredly, a better understanding of the mechanisms by which *C. albicans* and other pathogenic yeasts adhere to human epithelium could serve as a critical first step in the prevention of disseminated disease in an enlarging spectrum of susceptible hosts.

Methods

Growth and radiolabeling of yeast isolates. Organisms used in these experiments were isolated in pure culture from blood (*Candida albicans* [eight isolates], *Candida tropicalis* [three isolates]) of hospitalized patients and were identified in the Clinical Microbiological Laboratory of the University of Minnesota Hospital by morphology, germ tube expression, and multiple assimilation substrates on a yeast biochemical card (Automicrobic System, Vitek, Inc., Cleveland, OH). Growth curves for each isolate were determined spectrophotometrically by absorbance at 420 nm with simultaneous colony counts. Yeast cells were grown to exponential phase at 24°C in Sabouraud's broth (Difco Laboratories, Inc., Detroit, MI) and were aliquoted and frozen at −70°C until use.

For each experiment, an aliquot of the desired isolate was grown overnight at 24°C with rotation in a defined medium containing albumin, amino acids, and 20 mM D-glucose (23). The following morning, yeast cells were pelleted; microscopic examination confirmed the presence of yeast-phase organisms without germ tubes or pseudohyphae. For unlabeled yeast cells, 50 μl of each pellet was inoculated into 5 ml of defined medium and grown to exponential phase (~ 4 h, rotating at 24°C). For labeled yeast cells, 50 μl of each pellet was inoculated into 5 ml of defined medium in which the methionine concentration had been reduced from 1.2 mM to 8.7 μM. 4 μl of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL; sp act > 800 Cu/mmol) were then added, and the organisms were grown to exponential phase as described above. Organisms were washed five times with PBS to remove free isotope and then diluted to an absorbance of 0.5 at 420 nm, which corresponded to a concentration of 5×10^7 CFU/ml for *C. albicans* and 2.5×10^7 CFU/ml for *C. tropicalis*.

Flow cytometry. Methods used to quantitate the expression of the integrin analogue on *C. albicans* have been previously described by our laboratory (14, 16, 23). Briefly, 5×10^6 CFU of each isolate were incubated in suspension with 60 μg OKM1 (1,200 μg/ml in 50 μl); with the irrelevant IgG_{2b} monoclonal MY9 (Coulter Immunology, Hialeah, FL) in the identical concentration; or with 50 μl of PBS. OKM1 (IgG_{2b}) was purchased as a hybridoma (American Type Culture Collection, Rockville, MD; ATCC CRL 8026) and was purified from supernatant by affinity chromatography on Protein A Sepharose CL4B (Pharmacia, Inc., Piscataway, NJ). SDS-PAGE under reducing conditions verified the presence of heavy and light chains at 50 and 25 kD, respectively.

Flow cytometry was performed on a FACStar Plus (Becton-Dickinson Immunocytometry Systems, Mountain View, CA). Fluorescence of control samples, in which the primary antibody was omitted, was then subtracted channel by channel to give the value designated as specific fluorescence. A positive yeast cell exhibited an increase in fluorescence intensity above autofluorescence, while the signal from a negative cell was no greater than that of the autofluorescent control. For each sample, we report both the mean channel fluorescence (MCF) and the percentage of 20,000 organisms exhibiting specific fluorescence with OKM1, after subtraction of nonspecific autofluorescence in the absence of the primary antibody. The IgG_{2b} control mAb MY9 failed to fluoresce above background; only 2.7±1.2% of *C. albicans* yeast cells fluoresced with an MCF of 202.0±31.6, while 2.6±1.3% of *C. tropicalis* yeast cells fluoresced with an MCF of 192.3±40.4. Flow cytometric analysis was performed on all isolates of *C. albicans* and *C. tropicalis*. On a given day, at least three isolates chosen at random underwent flow cytometry; blood isolate 315, which we have previously described (23), was used as a control in all experiments because of its reproducible fluorescence with OKM1 (77.6±1.4% and MCF of 618.5±15.2 for $n = 23$ experiments).

Culture of the human epithelial cell line. HeLa S3, a cervical epithelioid carcinoma cell line (ATCC CCL 2.2), was maintained in continuous culture in Ham's F-12 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. For use in the adhesion assay, 1 ml of epithelial cells at a concentration of 4×10^5 cells/ml was inoculated into each well of a 24-well plate (Costar Data Packaging Corp., Cambridge, MA), and grown over 48 h to a confluent monolayer in the same medium. Confluence was assessed by visual inspection using a Nikon inverted microscope.

Adhesion assay. The adhesion assay was modified from our previously published methods for adhesion to human umbilical vein endothelium (23). A representative isolate of each *Candida* species was grown in the defined medium, labeled with [^{35}S]methionine, washed five times in PBS, and resuspended in HBSS with calcium and magnesium, to which was added 1% BSA (HBSS^{++}/BSA). 5×10^4 CFU labeled yeast cells ($1.8–2.1 \times 10^5$ cpm) were added to each well of the HeLa monolayers, with a final volume of 250 μL/well. For quantitation of nonspecific binding, a 100-fold excess (5×10^6 CFU) of unlabeled yeast cells was added to labeled yeast cells in one-third of the experimental wells. Experimental plates were then incubated for 1 h at 37°C in 5% CO_2. After the incubation period, each plate underwent microscopic examination to confirm that yeast morphology was maintained (< 5% germ tubes present for *C. albicans*). The supernatant was removed from each well and the monolayers washed twice with HBSS^{++}/BSA to remove nonadherent yeasts. The supernatant and washes for each well were pooled, solubilized in Ecolume (ICN Biomedicals Inc., Irvine CA) and counted in a β-scintillation counter (LS230; Beckman Instruments, Inc., Palo Alto, CA). The epithelial monolayers and adherent yeast cells were removed with a commercial preparation of trypsin/EDTA (Life Technology, Inc.) and 0.5 M NaOH, then solubilized in Ecolume and counted in a β-scintillation counter. Duplicate wells were assayed for each sample. Loss of yeast cells was < 10% of the total cpm. Total adhesion was calculated according to the following formula: [cpm(adherent yeasts)/cpm(yeasts in supernatant) + (adherent yeasts)] × 100. Specific adhesion was calculated as the difference between total adhesion and nonspecific adhesion, where the latter was measured in the presence of a 100-fold excess of unlabeled yeast cells. Under these conditions, total cpm for specifically adherent yeasts ranged from 0.8 to 1.0×10^3.

Purification of iC3b, C3d, and fibronectin. Fig. 1 displays the structures of all purified proteins used in these experiments, as analyzed by SDS-PAGE under reducing conditions. Human C3 was isolated from fresh plasma after affinity chromatography on Sepharose L-lysine, ion exchange chromatography on DEAE-Sephacel, and sizing chromatog-

1. *Abbreviations used in this paper:* MCF, mean channel fluorescence; RAD, arginine-alanine-aspartic acid, RGD, arginine-glycine-aspartic acid; RGK, arginine-glycine-lysine.

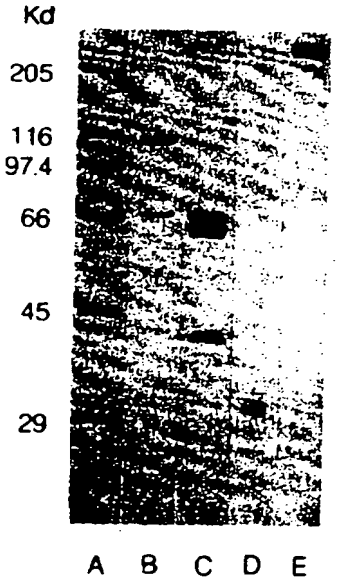

Figure 1. 10% SDS-polyacrylamide gel showing M_r markers (lane *A*) and purified proteins C3 (lane *B*), iC3b (lane *C*), C3d (lane *D*), and fibronectin (lane *E*), under reducing conditions.

raphy on Sepharose CL6B, according to the method of Tack et al. (25). The C3 fragments iC3b and C3d were then isolated according to published methods (18, 26). Fibronectin was purified from plasma by chromatography on gelatin-Sepharose (Pharmacia), according to the methods of Regnault et al. (27). The isolation of fibronectin was confirmed by Western blot analysis using goat antiserum to human fibronectin (Atlantic Antibodies, Stillwater, MN).

Peptides. A 15-mer peptide, EIATRYRGDQDATMS, which encompasses the RGD site in iC3b (amino acid residues 1387–1401) and a control 15-mer peptide with the substitution of arginine-glycine-lysine (RGK) for RGD, were the gifts of Dr. John Lambris (Department of Pathology, University of Pennsylvania, Philadelphia, PA). Flanking residues in both 15-mer peptides were identical to the iC3b sequence, save for the substitution of alanine for cysteine at amino acid residue 1389. Additional 100-mg quantities of the 15-mer RGD and RGK peptides were synthesized in the Microchemical Genetics Facility at the University of Minnesota on a model 9600 peptide synthesizer (MilliGen/Biosearch, Burlington, MA), with subsequent purification on a Spectrophysics HPLC (Hewlett-Packard Co., Palo Alto, CA). Amino acid analysis of the purified samples was done on a model 6300 amino acid analyzer (Beckman Instruments, Inc.) A series of smaller iC3b peptides—RGDQD, TRYRGD, TRYRGDQD, RGDQDATMS, TRYRGDQDAT, TRYRGKQD, and a 12-mer peptide, EIATRYQDATMS, which included only the flanking residues from the 15-mer peptide—were also synthesized by this facility. Two 7-mer peptides, GRGDSPG and GRADSPG (the former from within the cell-binding domain of fibronectin) were synthesized as above. PepTite 2000 (a proprietary ~ 23-mer peptide containing the amino acid sequence—XXXGRGDSPASSKXXX—surrounding the RGD site in native fibronectin) was purchased from Telios Pharmaceuticals, San Diego, CA (28).

Inhibition of adhesion with anti-α_M mAbs, iC3b, C3d, fibronectin, and peptides. Anti-Mo1, an IgM mAb, was obtained from Coulter Immunology. mAb 17 (IgM) and mAb 44 (IgG_{2b}) were the kind gifts of Dr. Robert Todd (University of Michigan, Ann Arbor, MI) and were dialyzed into PBS before use (29). OKM1 was purchased and purified as described above. Yeast cells were preincubated with the anti-α_M mAbs as previously described (23). Briefly, 5×10^6 [^{35}S]methionine-labeled yeast cells were incubated in suspension for 30 min at 4°C with either 400 μg/ml anti-Mo1, 2,000 μg/ml OKM1, 300 μg/ml mAb 17, or 300 μg/ml mAb 44; final volume was 50 μl. Yeasts were then pelleted, washed three times with PBS, and used in the adhesion assay described above.

Yeast cells were also preincubated with iC3b, C3d, fibronectin, or the synthetic peptides before use in the adhesion assay. 2.5×10^6 [^{35}S] methionine-labeled *C. albicans* were incubated for 45 min at 37°C on a rotating rack with purified human iC3b or purified human fibronectin at a concentration of 0.01 mM (1.8 mg/ml iC3b and 4.8 mg/ml fibronectin) in a final volume of 50 μl; each experimental well was inoculated with 5×10^4 labeled yeast cells. For wells used to quantitate nonspecific binding, a 100-fold excess (5.0×10^6) of unlabeled *C. albicans* preincubated in 0.02 mM iC3b or fibronectin was inoculated. As additional controls in the iC3b inhibition experiments, labeled and unlabeled *C. albicans* were incubated with BSA (Sigma Chemical Co., St. Louis, MO) or purified C3d (an iC3b degradation fragment that does not contain the C3 RGD site) at the concentration and conditions described above. For the peptide inhibition studies, preliminary dose-response experiments confirmed that 1.0 mg/ml was the optimal inhibitory concentration for all 12 peptides; all peptide inhibition experiments reported in this paper were therefore performed at this concentration. For studies with *C. tropicalis*, preincubation with purified human iC3b, purified human fibronectin, BSA or the synthetic peptides was performed in the same manner. For the experiments involving preincubation with multiple ligands, the yeast cells were first incubated with the specified peptide for 45 min at 25°C, and then for 45 min at 37°C with either iC3b or fibronectin, all at the same concentrations as described above. After preincubation, the adhesion assay and the calculation of specific adhesion were performed as previously described.

Immunofluorescence of HeLa cells. 2 ml of HeLa cells at a concentration of 4×10^5 cells/ml in Ham's F-12 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine were added to each well of a six-well tissue culture plate (Costar) containing a sterile glass coverslip. Plates were then incubated at 37°C, 5% CO_2 for 48 h. The cell-coated coverslips were washed twice with unsupplemented Ham's F-12 medium and incubated at 37°C, 5% CO_2 for 4 h. The cell-coated coverslips were then washed twice with PBS, fixed by submersion in cold 95% ethanol for 2 min, and washed an additional two times with PBS. Cell-coated coverslips were then incubated with one of the following primary antibodies diluted as indicated in PBS/3% BSA: 1:1,000 dilution of goat anti-human C3 (Atlantic Antibodies, Stillwater, MN), 1:10 dilution of mouse anti-human iC3b (Quidel, San Diego, CA), 1:100 dilution of goat anti-human fibronectin (Atlantic Antibodies), 1:100 dilution of goat anti-human neutrophil elastase (Behring Diagnostics, LaJolla, CA), or PBS/3% BSA alone, for 30 min at 37°C in 5% CO_2. The cell-coated coverslips were then washed three times with PBS/3% BSA and were incubated for 45 min at 37° in 5% CO_2, with a 1:100 dilution of either rabbit anti-goat IgG conjugated to fluorescein isothiocyanate (Kallestad, Austin, TX) or goat F(ab')$_2$ antibody to mouse IgM/IgG conjugated to fluorescein isothiocyanate (Tago Inc., Burlingame, CA), as appropriate for the primary antibody. The cell-coated coverslips were then washed three times with PBS, inverted onto a glass microscope slide pretreated with a drop of glycerol with 2.5% DABCO (Sigma) and examined under phase-contrast and immunofluorescent microscopy (Laborlux 12; E. Leitz, Inc., Rockleigh, NJ) at ×400.

Inhibition of adhesion with polyclonal anti-C3 and anti-fibronectin antibodies. Goat anti-human C3 (Atlantic Antibodies) and goat anti-human fibronectin (Atlantic Antibodies), were each dialyzed against PBS at 4°C to remove azide. HeLa cell monolayers were washed twice with $HBSS^{++}$, once with $HBSS^{++}$/BSA, and then incubated with either a 1:2 dilution of the specified antibody solution in $HBSS^{++}$/BSA or $HBSS^{++}$/BSA alone, in a final volume of 250 μl for 60 min at 37°C. The antibody solution was then aspirated off the monolayers which were then washed twice with $HBSS^{++}$/BSA and incubated with ^{35}S-labeled *C. albicans* or *C. tropicalis* in the adhesion assay.

Statistics. Results, expressed as mean±standard error unless otherwise noted, were analyzed using the Student's *t* test for unpaired samples. $P < 0.05$ was considered significant.

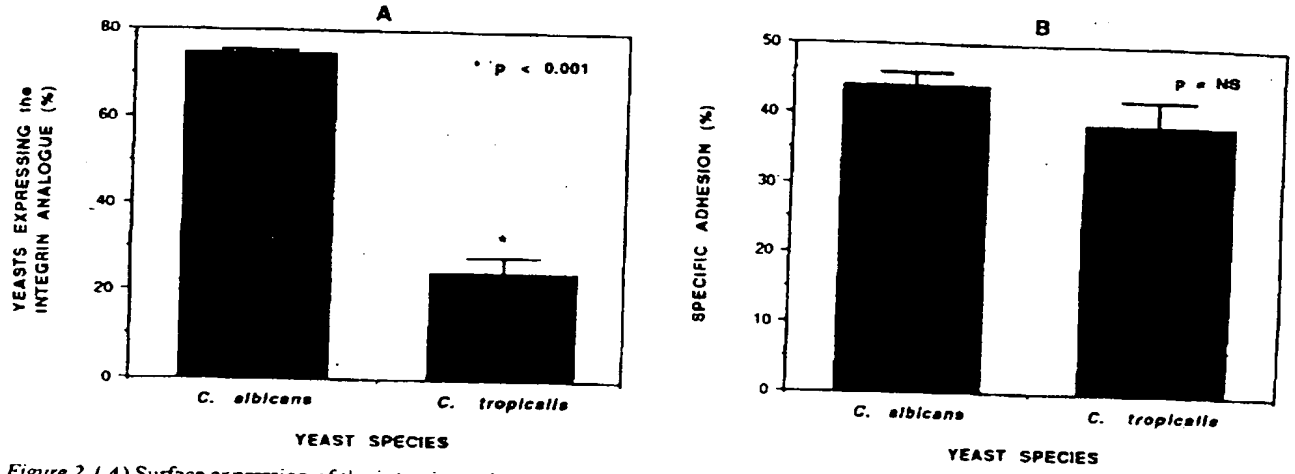

Figure 2. (*A*) Surface expression of the integrin analogue in *C. albicans* and *C. tropicalis* as determined by flow cytometry with the monoclonal antibody OKM1, shown as percent yeasts fluorescing. Results represent the mean±standard error of $n \geq 3$ experiments with each isolate. As indicated in the methods, multiple isolates of each species were tested. (*B*) Specific adhesion (%) of *C. albicans* and *C. tropicalis* isolates to the human epithelial cell monolayers (Hela S3) in vitro. Results represent the mean±standard error of $n = 9$ experiments.

Results

Surface expression of the integrin analogue and adhesion to human epithelium in C. albicans and C. tropicalis. Fig. 2 *A* compares surface expression of the integrin analogue in *C. albicans* and *C. tropicalis*, as quantitated by flow cytometry with saturating concentrations of the mAb OKM1. Expression of the integrin analogue was significantly greater in *C. albicans*, with 74.5±1.0% of yeast cells fluorescing and an average MCF of 614.3±11.6. For *C. tropicalis*, only 24.2±3.8% of the yeast cells fluoresced under the same conditions ($P < 0.001$), with an MCF of 303.5±25.7 ($P < 0.001$). Thus, surface expression of the integrin analogue is higher in *C. albicans* and is significantly reduced in *C. tropicalis*.

In contrast, Fig. 2 *B* demonstrates that epithelial adhesion did not differ between *C. albicans* and *C. tropicalis* (44.0±1.9% for *C. albicans* vs. 38.8±3.6% for *C. tropicalis* [$P = 0.180$]). These results prompted us to examine the role of the integrin analogue in epithelial adhesion in *C. albicans* and to search for a different mechanism of adhesion in *C. tropicalis*.

Inhibition of epithelial adhesion of C. albicans with anti-α_M mAbs. Preincubation of *C. albicans* with mAb 17 (IgM) and mAb 44 (IgG_{2a}) inhibited specific adhesion of *C. albicans* to the epithelial monolayer by 58.2% and 52.4%, respectively ($P \leq 0.005$) (Fig. 3). mAbs anti-Mol (IgM) and OKM1 (IgG_{2b}) did not inhibit epithelial adhesion of *C. albicans*. We have previously shown that mAbs 17 and 44 significantly decrease the adhesion of *C. albicans* to human umbilical vein endothelium, while anti-Mol is ineffective in this regard (23). The inhibitory effects of mAbs 17 and 44 suggest that epithelial adhesion in *C. albicans* is epitope-specific.

Inhibition of epithelial adhesion of C. albicans with purified proteins. The integrin α_M/β_2 recognizes RGD sites in at least two ligands: iC3b and fibrinogen (19, 30). In addition, a fibronectin receptor in *C. albicans* has been implicated in endothelial adhesion (28, 31–33). We therefore evaluated the ability of purified iC3b and fibronectin to inhibit epithelial adhesion in *C. albicans*. Controls for these experiments included the non-RGD-containing proteins BSA and C3d. As shown in Fig. 4, adhesion of *C. albicans* preincubated with buffer alone was 43.3±4.3%, while adhesion of yeast cells preincubated with BSA, C3d, or fibronectin was not significantly reduced. Adhesion of *C. albicans* preincubated with iC3b was reduced by 76.1% ($P < 0.001$). These results confirm that a significant proportion of epithelial adhesion in *C. albicans* is mediated by the integrin analogue and can be inhibited by its ligand, iC3b.

Inhibition of epithelial adhesion of C. albicans with synthetic peptides. The effects of synthetic peptides on candidal adhesion are shown in Table I. Two types of peptides were constructed and tested; group I peptides are derived from the RGD-site in iC3b, while group II peptides are derived from the RGD-site in fibronectin. Among the iC3b peptides, no significant inhibition of *C. albicans* adhesion was found with peptides ≤ 8-mer or with either the 8-mer or the 15-mer RGK

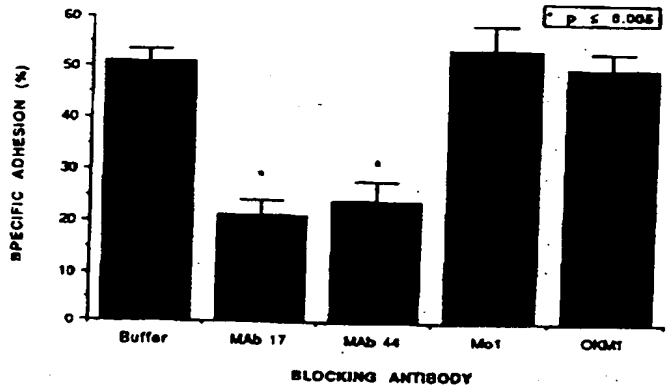

Figure 3. Effect of preincubation with the anti-α_M mAbs mAb 17, mAb 44, anti-Mol, and OKM1 on specific adhesion of *C. albicans* to human epithelial monolayers. Results represent the mean±standard error of $n = 3$ experiments.

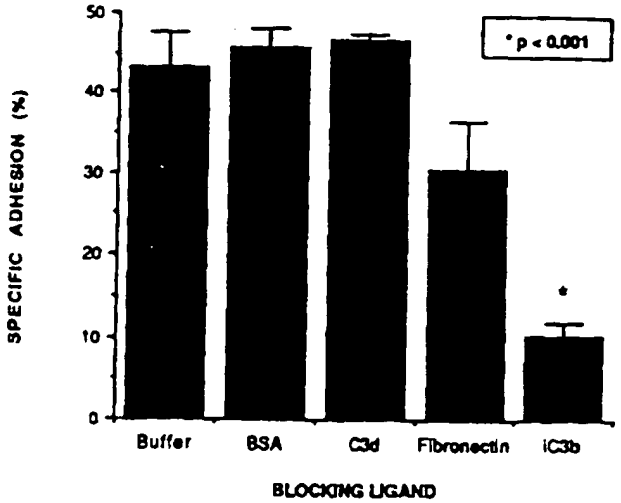

Figure 4. Effect of preincubation with buffer, BSA, C3d, fibronectin, and iC3b on specific adhesion of *C. albicans* to human epithelial monolayers. Results represent the mean±standard error of $n = 3$ or 4 experiments with each purified protein.

peptides. However, *C. albicans* adhesion to human epithelium was inhibited by 21.3% after pretreatment with the 9-mer RGD peptide ($P = 0.027$), by 35.6% with the 10-mer RGD peptide ($P = 0.001$), and by 49.7% with the 15-mer RGD peptide ($P < 0.001$).

Two pieces of evidence indicate the importance of the flanking sequence in inhibition of epithelial adhesion. Firstly, the 12-mer peptide EIATRYQDATMS, which contains those sequences flanking the RGD site in iC3b, inhibited epithelial adhesion of *C. albicans* by 21.4% ($P = 0.034$). Secondly, the 15-mer RGK peptide, which also contains these flanking sequences, diminished *C. albicans* adhesion by ~ 20%

Table I. Effects of Synthetic Peptides on Epithelial Adhesion of C. albicans

Peptide	Specific adhesion*	Inhibition of adhesion	P value
	%	%	
Group I—iC3b peptides			
Buffer alone	44.1±2.3		
RGDQD	47.1±1.6	−6.8	NS
TRYRGD	46.9±0.9	−6.2	NS
TRYRGDQD	45.1±1.5	−2.1	NS
RGDQDATMS	34.8±1.4	21.3	0.027
TRYRGDQDAT	28.5±1.4	35.6	0.001
EIATRYRGDQDATMS	22.2±2.9	49.7	<0.001
EIATRYQDATMS	32.9±3.7	21.4	0.034
EIATRYRGKQDATMS	35.3±4.6	20.1	NS
TRYRGKQD	45.3±2.1	−2.7	NS
Group II—fibronectin peptides			
Buffer alone	39.0±2.3		
GRGDSPG	36.8±2.4	5.7	NS
PepTite 2000	44.3±2.5	−13.5	NS
GRADSPG	35.5±1.7	8.9	NS

* Results expressed as the mean±SE, n = 3–9 experiments.

Table II. Competitive Effect of iC3b and a Noninhibitory Peptide on Epithelial Adhesion of C. albicans

Pretreatment	Specific adhesion*	Inhibition of adhesion	P value
	%	%	
Buffer	43.8±1.2		
iC3b	12.7±1.3	81.1	<0.001
TRYRGDQD followed by iC3b	36.4±2.2	17.1	0.041

* Results expressed as the mean±SE, $n = 3$ experiments.

(35.3±4.6%), although this reduction was not statistically significant. In contrast, none of the group II, or fibronectin, peptides significantly inhibited the adhesion of *C. albicans* to human epithelium.

These results show that a 9-mer sequence encompassing the RGD-site and flanking residues in iC3b is sufficient for inhibition of *C. albicans* adhesion, but that maximal inhibition requires a longer flanking sequence (15-mer RGD peptide). The degree of inhibition observed with peptides containing iC3b flanking sequences, as well as the lack of inhibition with fibronectin peptides, support the contention that the amino acids surrounding the RGD site are also important in mediating adhesion of *C. albicans* to human epithelium. Such flanking sequences may provide specificity for recognition of target ligands by the integrin analogue in *C. albicans*.

Competitive inhibition studies provide additional evidence that the integrin analogue in *C. albicans* recognizes the RGD site in iC3b (Table II). Preincubation of *C. albicans* with the 8-mer noninhibiting RGD-peptide nullified the inhibitory effect of iC3b (from 81.1% with iC3b alone to 17.1% after preincubation with the smaller peptide) ($P = 0.001$). These results confirm that the inhibition observed with iC3b is attributable to its RGD sequence and flanking residues, and not to charge or steric hindrance.

Inhibition of epithelial adhesion of C. tropicalis with purified proteins. Despite a low level of surface expression of the integrin analogue (24.2±3.8%, Fig. 2 A), *C. tropicalis* did not differ significantly from *C. albicans* in adhesion to human epithelial monolayers in vitro (Fig. 2 *B*). Therefore, in a separate series of experiments, we compared the effect of iC3b and fibronectin on the adhesion of *C. tropicalis* (Fig. 5). In contrast to results obtained with *C. albicans*, preincubation of *C. tropicalis* with fibronectin reduced adhesion by 53.9% ($P = 0.017$), while iC3b was wholly ineffective. These results confirm that *C. tropicalis*, in which surface expression of the integrin analogue is significantly reduced, does not recognize iC3b as a ligand for adhesion.

Inhibition of epithelial adhesion of C. tropicalis with synthetic peptides. Table III shows the effects of synthetic peptides on the adhesion of *C. tropicalis*. As predicted from the studies with purified iC3b (Fig. 5), pretreatment of *C. tropicalis* with iC3b-RGD peptides and their controls (group I) did not significantly inhibit epithelial adhesion. Conversely, preincubation of *C. tropicalis* with either of two RGD peptides from fibronectin, a proprietary ~ 23-mer peptide (PepTite 2000) and a 7-mer peptide, decreased specific adhesion by 39.5% and 22.1%,

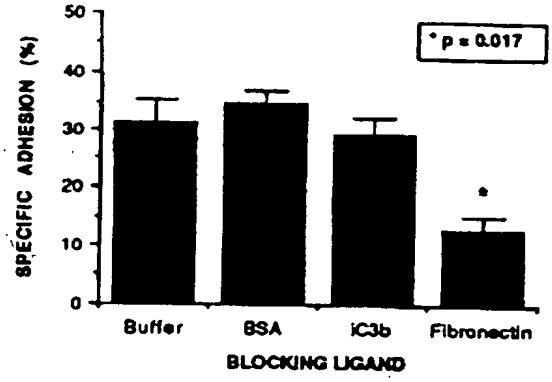

Figure 5. Effect of preincubation with buffer, BSA, iC3b, and fibronectin on specific adhesion of *C. tropicalis* to human epithelial monolayers. Results represent the mean±standard error of $n = 3$ experiments.

respectively ($P = 0.021$ for the 23-mer and $p = 0.003$ for the 7-mer). Preincubation with a 7-mer arginine-alanine-aspartic acid (RAD) peptide had no effect. These results implicate the RGD site in fibronectin as the ligand for epithelial adhesion in *C. tropicalis*.

Competitive inhibition studies provide further support for this hypothesis, as shown in Table IV. Preincubation of *C. tropicalis* with the 7-mer fibronectin peptide before incubation with fibronectin itself reduced the inhibitory effects of fibronectin from 50.9% (fibronectin alone) to 16.7% ($P = 0.002$).

Immunofluorescence of HeLa cells. If RGD-containing ligands such as iC3b and fibronectin are target sites for epithelial attachment of candidal species, then they must be present at the surface of epithelial cells. Fig. 6 displays the immunofluorescent micrographs obtained when epithelial monolayers (cultured under serum-free conditions for 4 h) were incubated with a polyclonal antibody to C3 (panel *A*); a monoclonal antibody to a neoantigenic determinant in iC3b which is absent in native C3, C3b, and C3d (panel *B*) (34); a polyclonal antibody to fibronectin (panel *C*); a polyclonal antibody to neutrophil elastase (panel *D*); and no primary antibody (panel *E*). It should be noted that the polyclonal antibody to C3 (panel *A*) recognizes the native protein as well as its degradation fragments C3b, iC3b, and C3d. Therefore, HeLa cells exhibit specific immunofluorescence with antibodies recognizing iC3b and fibronectin, the ligands implicated in the epithelial adhesion of *C. albicans* and *C. tropicalis*.

Table III. Effects of Synthetic Peptides on Epithelial Adhesion of C. tropicalis

Peptide	Specific adhesion*	Inhibition of adhesion	*P* value
	%	%	
Group I—iC3b peptides			
Buffer alone	43.7±2.5		
RGDQD	33.2±5.6	24.1	NS
TRYRGD	42.4±5.3	3.0	NS
TRYRGDQD	40.0±5.2	8.5	NS
RGDQDATMS	39.2±1.8	10.3	NS
TRYRGDQDAT	47.3±3.7	−8.2	NS
EIATRYRGDQDATMS	42.1±2.6	3.7	NS
EIATRYQDATMS	44.1±3.4	−1.0	NS
EIATRYRGKQDATMS	42.5±3.7	2.8	NS
TRYRGKQD	39.9±4.1	8.7	NS
Group II—fibronectin peptides			
Buffer alone	43.6±1.9		
GRGDSPG	34.0±1.3	22.1	0.003
PepTite 2000	26.4±6.5	39.5	0.021
GRADSPG	43.1±2.3	1.1	NS

* Results expressed as the mean±SE, $n = 3$–5 experiments.

Table IV. Competitive Effect of Fibronectin and a Noninhibitory Peptide on Epithelial Adhesion of C. tropicalis

Pretreatment	Specific adhesion*	Inhibition of adhesion	*P* value
	%	%	
Buffer	39.1±1.0		
Fibronectin	19.2±1.0	50.9	<0.001
GRGDSPG followed by fibronectin	32.6±1.5	16.7	0.021

* Results expressed as the mean±SE, $n = 3$ experiments.

Inhibition of candidal adhesion by pretreatment of epithelial monolayers with antibodies to C3 and fibronectin. Having identified those surface components of *C. albicans* and *C. tropicalis* that mediate epithelial adhesion, we next sought to confirm C3 and fibronectin as their respective targets on the epithelial cell. For *C. albicans*, pretreatment of the HeLa cell monolayer with a polyclonal antibody to C3 diminished adhesion by 27.3% ($P = 0.002$), while the anti-fibronectin antibody had no effect (Fig. 7). The opposite results were found with *C. tropicalis*: pretreatment with anti-fibronectin antibodies reduced adhesion by 31.5% ($P = 0.004$), while anti-C3 antibodies were ineffective.

Discussion

Clinical experience attests to the ready adhesion of *C. albicans* to epithelial cells in the gastrointestinal and genitourinary tracts. In vitro, *Candida* species also adhere to a variety of epithelial cells, including exfoliated buccal and vaginal epithelial cells (9, 10, 35–42), as well as cultured cell lines such as HeLa S3, a cervical carcinoma (43). A number of experimental observations suggest that adhesins in *C. albicans* are surface mannoproteins which are in turn induced by glucose, increased in expression in germinated cells, and inhibited by proteolytic enzymes (11, 35–42). Hydrophobic interactions between candidal surface proteins and the host epithelial cell have also been implicated (44), as have been a variety of matrix proteins including fibronectin, laminin, and fibrinogen (31–33, 45–48). Binding sites for these extracellular matrix proteins have also been described on germ tubes or mycelia of *C. albicans* (46, 47), but their presence in yeast-phase organisms is debated. *C. albicans* mutants that lack surface proteins

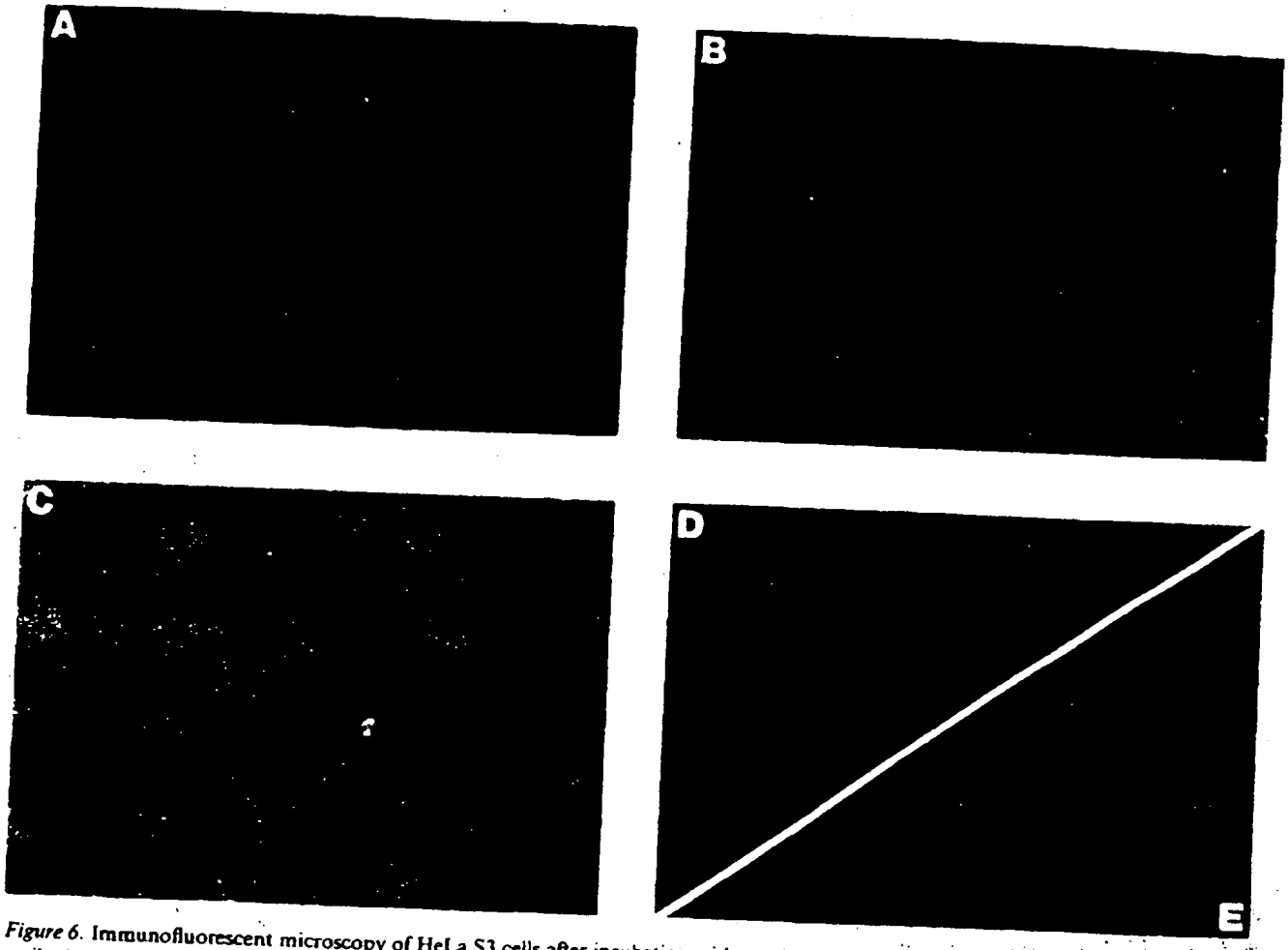

Figure 6. Immunofluorescent microscopy of HeLa S3 cells after incubation with a polyclonal antibody to human C3 (*A*), a murine monoclonal antibody against iC3b (*B*), a polyclonal antibody to human fibronectin (C), or a polyclonal antibody to human neutrophil elastase (*D*). In *E*, PBS/3%BSA was substituted for the primary antibody. These cells, cultured on glass coverslips, failed to form confluent monolayers, as compared to HeLa cells cultured in plastic microtiter plates.

of 50–60 and 165 kD are less adherent to buccal epithelial cells (42). In comparative studies with human or porcine endothelium, *C. albicans* and *C. tropicalis* were generally more adherent than other *Candida* species or *S. cerevisiae* (49, 50), an indication that adhesion may well correlate strongly with pathogenicity.

As previously shown by us, expression of the integrin analogue in *C. albicans* is induced by glucose, inhibited by proteolytic enzymes, and exponentially increased after germination (14, 16). Moreover, because of its antigenic, structural, and functional homologies with α_M and α_X, the integrin analogue in *C. albicans* also serves to mediate yeast adhesion to human endothelial cells (23). Given these characteristics, the role of the integrin analogue as an epithelial adhesin is clearly deserving of study.

In mammalian cells, the β_2-integrins regulate the adhesion of leukocytes by recognition of the tripeptide sequence RGD in proteins of the extracellular matrix (19–21). The ligand specificity of the various integrins is based in part on the presence of differing flanking residues surrounding the RGD sequence in these matrix proteins (20, 21). Our data support a similar role for the integrin analogue in *C. albicans* in mediating adhesion of this yeast to human epithelium. The polypeptide iC3b reduced the adhesion of *C. albicans* to human epithelium by 76%, while C3d (which does not contain the RGD site) and fibronectin (which does not contain the appropriate flanking residues) were ineffective. More precisely, 9-mer, 10-mer, and 15-mer peptides containing the iC3b RGD sequence with the appropriate flanking residues, and a 12-mer peptide consisting of only the flanking amino acids, significantly reduced adhesion by 21–50%. Maximal inhibition of adhesion to human epithelium was achieved with the 15-mer RGD peptide. Peptides less than eight amino acids in length, RGK-substituted peptides, and fibronectin-based RGD peptides without the appropriate iC3b flanking residues did not inhibit epithelial adhesion in *C. albicans*. Indeed, the 7-mer noninhibitory peptide used in these experiments is derived from the RGD site in fibronectin and has previously been reported to limit tissue dissemination in a murine model of *C. albicans* fungemia (51). Taken together, these results suggest that the integrin analogue in *C. albicans*, like its mammalian counterpart, recognizes both the RGD tripeptide and specific flanking sequences in iC3b; the inefficacy of the 7-mer fibronectin peptide emphasizes that there are critical distinctions in the mechanisms by which *C. albicans* adheres to epithelium, as opposed to endothelium.

In several clinical studies f fungemia, *C. tropicalis* is the second most frequently isolated species (3, 8). By flow cytome-

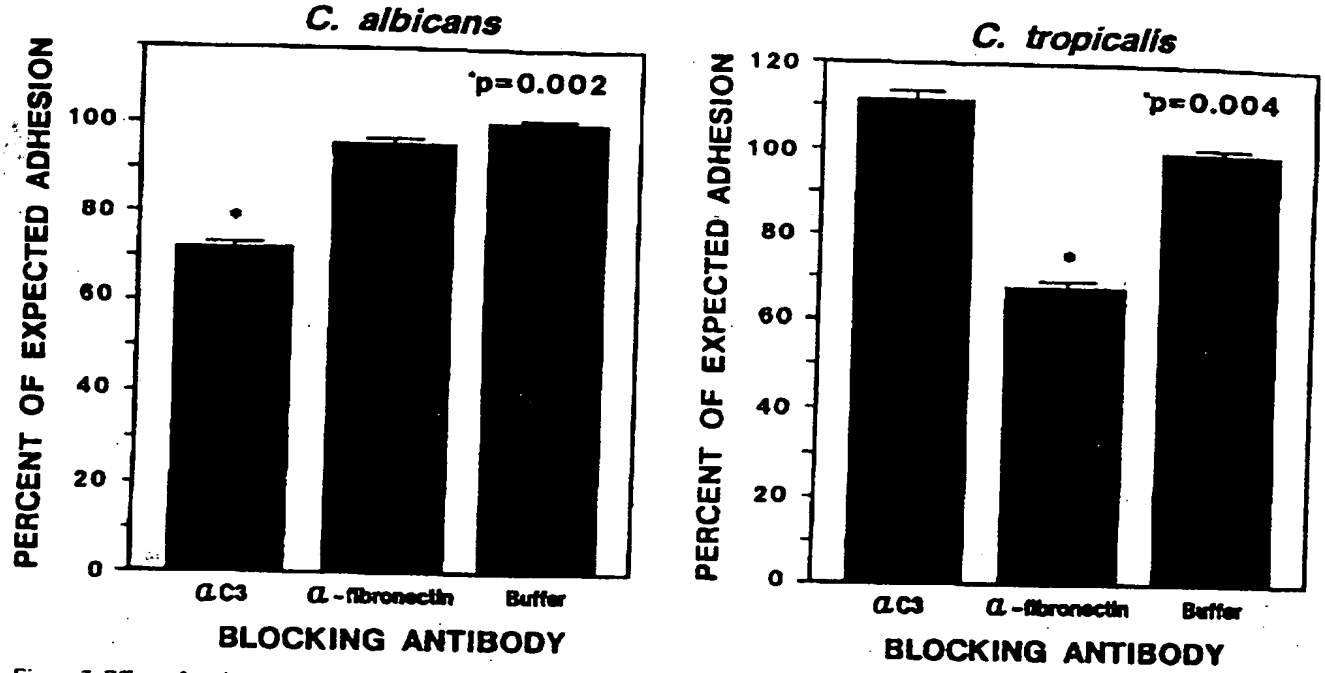

Figure 7. Effect of preincubation of the epithelial monolayer with buffer, goat anti-human C3, and goat anti-human fibronectin on specific adhesion of *C. albicans* and *C. tropicalis*. Results represent the mean±standard error of $n = 4$ experiments.

try, surface expression of the integrin analogue in *C. tropicalis* was low at 24.2±3.8% (Fig. 2 *A*), and yet this yeast did not differ from *C. albicans* in its adhesion to human epithelium (Fig. 2 B). Although iC3b and iC3b-RGD peptides did not significantly inhibit epithelial adhesion of *C. tropicalis* (Fig. 5 and Table III), two peptides in group I—RGDQD and RGDQDATMS—were more inhibitory than the remainder, although their effects were variable (P values of 0.163 and 0.213, respectively). It is intriguing that these two peptides differ from the other group I peptides in the absence of a specific leftward flanking sequence, which distinction may be the source of their relatively more inhibitory effects for *C. tropicalis*.

In contrast to the effects of iC3b and iC3b-derived peptides on *C. albicans*, *C. tropicalis* appears to have developed an alternative mechanism of adhesion by recognition of fibronectin (Fig. 5). Fibronectin reduced the epithelial adhesion of *C. tropicalis* by 58%, while fibronectin-derived RGD peptides inhibited adhesion by 22–40% (Table III). The smaller 7-mer fibronectin peptide—GRGDSPG—was minimally inhibitory at 22.1% ($P = 0.003$), while the larger 23-mer peptide (containing more of the appropriate fibronectin flanking sequences) had a greater effect (39.5% inhibition, $P = 0.021$). These results suggest that the degree of inhibition of *C. tropicalis* adhesion may be correlated with the number of specific amino acids to the left of the RGD sequence in the fibronectin peptides. Because a fibronectin receptor in *C. albicans* has recently been identified by ligand-binding studies (33), characterization of a putative fibronectin receptor in *C. tropicalis*, delineation of its relationship to the β_1-integrins, and more definitive studies of its role in epithelial adhesion are clearly warranted.

For both *C. albicans* and *C. tropicalis*, double-blockade experiments demonstrated that preincubation with small RGD peptides (7- or 8-mer) was sufficient to nullify the effects of the larger ligands, either iC3b for *C. albicans* or fibronectin for *C. tropicalis* (Tables II and IV). These results underscore the importance of the RGD-flanking sequence as a regulator of species-specific adhesion.

Lastly, our immunofluorescent micrographs have indicated that both iC3b and fibronectin, two substrates for candidal adhesion, are present at the surface of the epithelial monolayer. These results confirm previous observations of C3 synthesis in normal human keratinocytes and the epithelial cell lines A-431 (human epidermoid carcinoma), A-549 (human pulmonary carcinoma), and Hep G2 (human hepatocellular carcinoma) (52–55). Moreover, ELISA assay and immunoprecipitation in our laboratory have confirmed the synthesis of C3 and its subsequent cleavage to iC3b by HeLa cells in serum-free medium (E. J. Michael and M. K. Hostetter, submitted for publication). Fibronectin, in turn, is synthesized by rat small intestine and is a well-established constituent of subendothelial extracellular matrix (20, 56, 57). As we demonstrated, antibodies against iC3b inhibit epithelial adhesion in *C. albicans*, while anti-fibronectin antibodies have the identical effect for *C. tropicalis*.

In conclusion, these findings confirm that iC3b is the epithelial ligand for the integrin analogue in *C. albicans*, which recognizes the RGD tripeptide and definitive iC3b flanking sequences. Peptides encompassing the RGD site in iC3b and at least three flanking residues on either side inhibit this interaction. In contrast, fibronectin is the target protein for epithelial attachment of *C. tropicalis*, the second most prominent pathogenic isolate; fibronectin peptides are most effective in inhibiting this interaction. Understanding the mechanisms of epithelial adhesion in vitro may permit the design of highly specific peptides to inhibit epithelial adhesion of pathogenic *Candida* species in vivo, thereby enabling us t avert or at least to dimin-

ish the colonization of patients with *Candida* species and the subsequent morbidity and mortality due to invasive infections with these organisms.

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